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6. AUTHOR(S)

Stice, Steven L.
Chilton, Jamie

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ArunA Biomedical, Inc.
425 River Road
Athens, GA 30602

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Director, Naval Research Lab
Attn: Code 5596
4555 Overlook Avenue, SW
Washington, D.C. 20375-5320

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Carbohydrates corresponding to PhaL, VVA, DBA, LTL and PNA lectin binding are up regulated upon differentiation of hESCs. In particular, VVA, DBA, LTL and PNA lectin binding increases in hNPCs but not in hMPCs. This suggests that these carbohydrates might be specific for function of hNPCs and could be used for enrichment of hNPCs from a mixed population of cells. In future work VVA and PNA lectins will be used to isolate a pure population of HNPCs and used for developing a neural progenitor cell based biosensor.

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Athens, GA 30605

Quarterly Report
Human Neural Cell-Based Biosensor

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Attn: Code 5596
4555 Overlook Avenue, SW
Washington, D.C. 20375-5320

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Submitted by:
Dr. Steven L. Stice, Principle Investigator
ArunA Biomedical, Inc.
425 River Road
Athens, GA 30602
Phone: 706-583-0071
Fax: 706-262-2821
Email: sstice@arunabiomedical.com

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Technical Report:

Human Neural Cell-Based Biosensor

Abstract

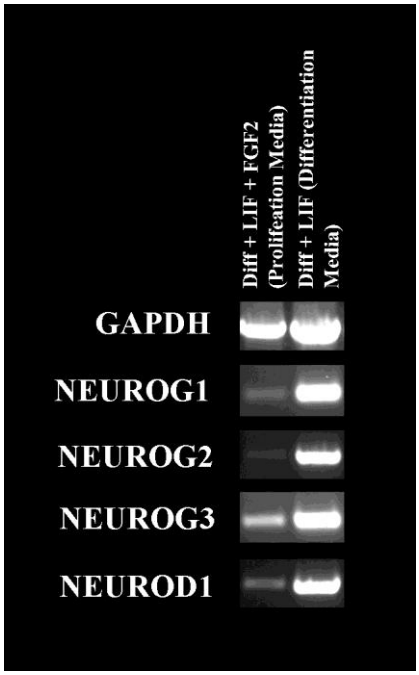
Carbohydrates corresponding to PhaL, VVA, DBA, LTL and PNA lectin binding are up regulated upon differentiation of hESCs. In particular, VVA, DBA, LTL and PNA lectin binding increases in hNPCs but not in hMPCs. This suggests that these carbohydrates might be specific for function of hNPCs and could be used for enrichment of hNPCs from a mixed population of cells. In future work VVA and PNA lectins will be used to isolate a pure population of HNPCs and used for developing a neural progenitor cell based biosensor.

bHLH over expressing cell lines:

Neural progenitors and partially differentiated neural progenitors were evaluated for expression levels of the pro-neuronal genes Neurogenin1, Neurogenin2, Neurogenin3 and NeuroD1.

Methods:

hNP cells were expanded as monolayer culture in proliferation media (defined as basal media and supplements, additionally supplemented with 10nG/mL FGF2) and split into two groups. Group one remained on in proliferation media, while the other group was cultured in differentiation media (defined as basal media and supplements, no FGF2). After 4 days cells were lysed and total RNA was extracted using the Qiagen RNAeasy Plus Kit, following standard protocol. Quantity and quality of RNA was assessed using spectrophotometry on Nanodrop 8000 (Thermo Scientific) spectrophotometer. Subsequently 1uG mRNA for each condition was converted to cDNA using the Applied Biosystems High Capacity cDNA synthesis kit.



To assay for gene expression PCR reactions were performed using the 20nG of each cDNA sample described above for each reaction. Primers were designed to specifically amplify endonegous human genes and not amplify products of the expression constructs to be used for transduction experiments. This will enable us to distinguish between and measure changes in exogenous and ectopic expression in future experiments.

Results:

Expression of endogenous Neurogenin 1 (NEUROG1), Neurogenin2 (NEUROG2), Neurogenin3 (NEUROG3) and NeuoD1 (NEUROD1) were upregulated at the end of four days in differentiation media.

This suggests that the hNP cells readily start to differentiate when FGF2 is withdrawn from culture, and effects of further upregulation of the expression of proneuronal genes will be tested in the next set of experiments. Differentiated cells will be initially assessed for maturity and function by RT-PCR detection of genes that are known markers of synapse formation and function. Human gene specific primers were designed to detect all known transcript variants for each gene. The genes include: Psd-95 (DLG4), Syntaxin I (STX1A), Synaptophysin (SYP), SNAP25 (SNAP25), GluR1 (GRIA1) glutamate receptor, ionotropic, AMPA1, Nav1.2 (SCN2A), Nav1.6 (SCN8A), CaV 2.1 (CACNA1A), HERG (KCNH2), and KCC2 (SLC12A5).

Derivation of a pure population of Neural progenitor cells using Lectins:

Human stem cells (hESCs) are pluoripotent stem cells capable of giving rise to all the cell types in the human body, such as, human neural progenitor cells (hNPCs) and human mesenchymal progenitor cells (hMPCs). Human Neural progenitor cells are multipotent cells capable of giving rise to all the cell types of the nervous system. The cell surface glycoproteins of neural progenitors play a significant role in various functions, such as, maintenance of multipotency, cell renewal, and differentiation. For example, the role of Notch has been implicated in directing neural stem cells to a glial cell fate and also has been implicated in neural stem cell renewal. While these two findings may seem contradictory, glysosylation of Notch on different serine residues could cause different outcomes [Haltiwanger, 2002 #5; Shi, 2008 #7]. Thus, the complexity of the carbohydrate surface is being realized.

To develop a hNPCs-based biosensor a pure population of neural progenitors is desirable. Here, we are using lectins to isolate a pure population of hNPCs based on their cell surface glycoprotein expression. We and others have began to characterize the surface of hESCs [Venable, 2005 #24] and their differentiated progeny [Wearne, 2008 #26;Wearne, 2006 #25]. Using lectins to probe the surface of hESC, we characterized percentages and localization of lectin binding. Lectins are carbohydrate binding proteins that recognize diverse sugar structures. Here we used a panel of 8 lectins to investigate the carbohydrate expression on surface of hESCs, hNPs and hMPs. First, the hESCs, hNPs and hMPs were characterized for battery of pluripotent, neuronal and mesenchymal intracellular and extracellular markers [Shin, 2006 #23]{Boyd, 2009 #31}. Lectin binding percentages on the hESCs, hNPs and hMPs were determined by flow cytometry. Immunocytochemistry using the lectin panel was analyzed to determine localization of lectin binding within adherent populations of cells and to validate binding specificity of each lectin using appropriate competitive sugars.

We found a variety of lectins binding unique carbohydrate moieties were present on hNP cell surfaces (**Figure**). In contrast to previous work on hESCs, a general trend towards increased lectin binding to the hNP surface when compared to that of the hESC surface in earlier studies, suggesting increased glycosylation occurs during or upon differentiation in vitro. Also, we noticed increased binding of VVA, LTL, and PNA corresponding to an increase in N-acetyl-D-galactosamine, Neu5Ac residues, and complex N-type glycans containing β1-6 linked branches, respectively on hNP surfaces. Our findings demonstrate that there are many surface carbohydrate antigens that could be exploited to further characterize and define the hNP cell phenotype.

Summary:

Carbohydrates corresponding to PhaL, VVA, DBA, LTL and PNA lectin binding are up regulated upon differentiation of hESCs. In particular, VVA, DBA, LTL and PNA lectin binding increases in hNPCs but not in hMPCs. This suggests that these carbohydrates might be specific for function of hNPCs and could be used for enrichment of hNPCs from a mixed population of cells. In future work VVA and PNA lectins will be used to isolate a pure population of HNPCs and used for developing a neural progenitor cell based biosensor.

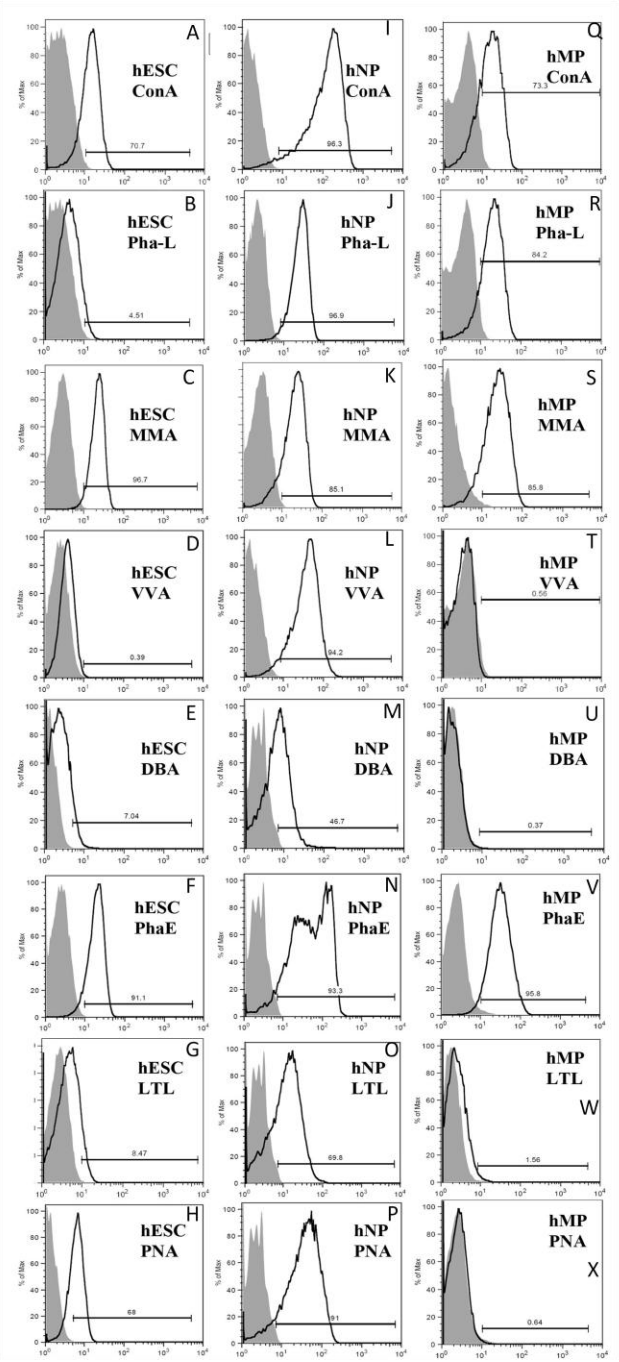


Figure: Flow cytometry histogram plots of lectin binding in hESCs, hNPs and hMPs. Percentage of cells binding to 8 different lectins was determined by flow cytometry**.** Panels A- H shows histograms of 8 different lectins binding to hESCs. Panels I- P shows histograms of lectin binding to hNPs; panels Q- X shows histograms of lectin binding to hMPs. A representative lectin histogram plot is shown from one of 3 experiments used in assessing lectin binding. Each far left grey fill peak in the histogram plot correlates with the secondary only stained cells and the shifted black tracing peak represent lectin binding.

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N00014-10-C-0066 Q1 Report

bHLH over expressing cell lines:

Current experiments underway in our lab include the optimization of methods to generate clonal cell lines from our human stem cell derived neural progenitor cells, or hNP cells. Such optimization is necessary as standard dilution assays for generating clones are unsuccessful with hNP cells. These hNP cells are extremely density-dependent for cell viability, health and ability to proliferate. The ability to create clonal lines will be useful in further studies for generating hNP cell lines expressing a specific bHLH factor with reduced variability for Tasks 1B and 1C. Previous studies have shown the successful use of ROCK inhibitor, a highly potent, cell-permeable selective inhibitor of Rho-associated protein kinase, to enhance the cloning efficiency of dissociated hESC without affecting their pluripotency. Thus, we hypothesized that treatment of hNPs with ROCK inhibitor would also assist in the generation of clonal hNP cell lines. To optimize generating hNP clonal lines, we explored the following questions:

1) What is the minimal cell plating density which allows neural progenitor survival without using any additional supporting reagents?
2) If utilizing a reagent such as ROCK inhibitor to prevent apoptosis and promote cell survival, what is the appropriate ROCK concentration to use?
3) What is the appropriate length of ROCK treatment?
4) If cells are first conditioned with ROCK treatment, can cell plating density be further reduced to generate clones?

In these studies, we observed from a cell plating density dilution assay in 96-well plates that the minimal cell plating density for hNP cells is 7,800 cells/cm^2 before viability and proliferation are severely and irreversibly compromised. To determine the appropriate ROCK inhibitor concentration to use, hNP cells were cultured in the presence of 5 µM, 10 µM, 20 µM or 50 µM ROCK inhibitor. Growth of hNP cells was enhanced with treatment with ROCK Inhibitor for all concentrations of ROCK inhibitor over CONTROL cells receiving no ROCK treatment. The hNP cells receiving ROCK treatment could also survive better at lower cell densities than CONTROL. We observed that hNP cells grew better at low densities in the presence of 10 µM ROCK inhibitor. Growth of hNP cells also benefitted greatly from continued culture in the presence of ROCK. Wells continuing to receive ROCK, instead of treatment stopping at 24 hrs, 2 days or 4 days, showed more growth (**Fig 1**). We further investigated whether hNP cells first conditioned in media

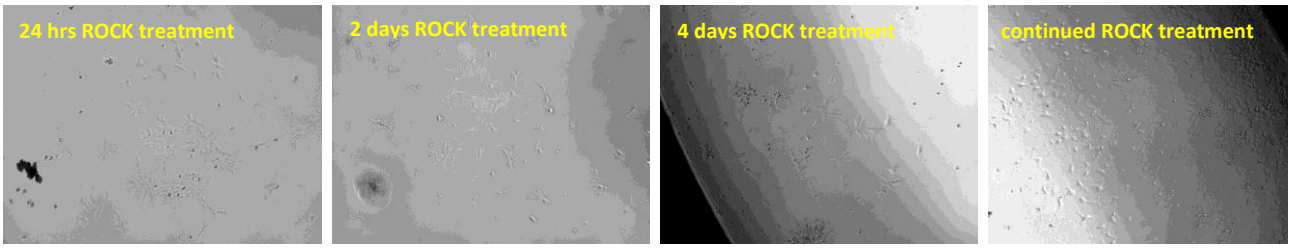

Fig 1: Phase contrast images (10X objective) at DAY 8 of hNP cells cultured in the presence of 50 µM ROCK Inhibitor for varying lengths of time (24 hrs, 2 days, 4 days and continued treatment for 8 days).

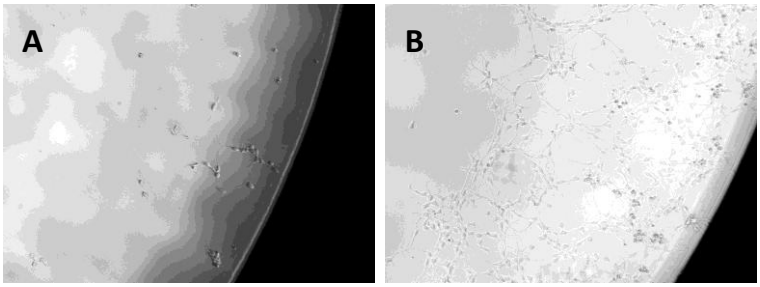

Fig 2: Phase contrast images (10X objective) of hNP cells after low density plating (~25 cells/well) in the presence of 10 µM ROCK inhibitor at DAY 2 (A) and after 10 days (B).

containing ROCK Inhibitor for 2 weeks could then be dissociated and proliferate from very low densities (~25 cells/well) in 96-well plates. After 10 days of ROCK treatment, these very low density wells showed substantial proliferation (**Fig 2**). We are currently dissociating these hNP cells again to potentially generate single cell clones by serial dilution.

Along with optimizing the generation of hNP clonal cell lines, we have been investigating the generation of neural progenitors derived from induced pluripotent stem cells, or iPSC-hNP cells. Considered by many to closely resemble human stem cells, iPSC are generated from somatic cells reprogrammed to an "induced pluripotent state" through ectopic expression of key transcription factors known to regulate pluripotency. Previously, ArunA has worked with Thermo Fisher Scientific to develop the viPS™ Vector Kit, commercially available since 2009, to generate induced pluripotent stem cells (iPSC) from human somatic cells. The viPS™ Vector Kit consists of multiple lentiviral vectors driven by the EF1α promoter, each encoding a different transcription factor known to generate iPSC via ectopic expression (OCT4, NANOG, SOX2, C-MYC, LIN28 and KLF4) (*1-3*). ArunA's iPSC consistently demonstrate those characteristics (i.e morphology, marker expression, in vitro differentiation plasticity, and in vivo teratoma formation) indicative of bona fide iPSC.

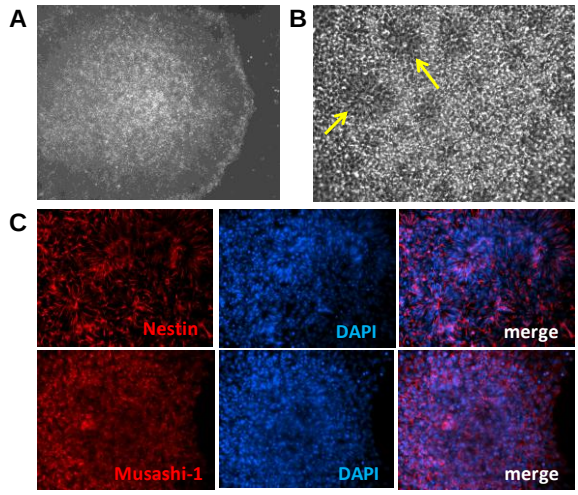

Fig 3: Differentiating iPSC form neural rosettes. (**A**) Starting population of pluripotent, feeder-free iPSC colonies (mTeSR™ 1); (**B-C**) Neural rosettes form within 14 days of culturing iPSC colonies in ArunA's derivation media and stain positive for nestin and musashi-1.

Subsequent derivation of neural progenitors from different sources of human iPSC may contribute added benefit in the development of a human neuron-based MEA biosensor, as it may provide valuable insight into possible gender and person-to-person variability in response to toxins. Our recent preliminary data suggests that ArunA's expertise in generating hNP cells as adherent monolayers is translatable to iPSC. The iPSC-derived neural rosettes are indistinguishable from hESC-derived neural rosettes in morphology, differentiation time, and early expression of nestin and musashi-1 (**Fig 3**). Furthermore, iPSC-hNP cells from iPSC-derived neural rosettes continue to show similar morphology, nestin and SOX2 (not shown) expression, and loss of OCT4 expression as their hNP cell counterparts (**Fig 4**).

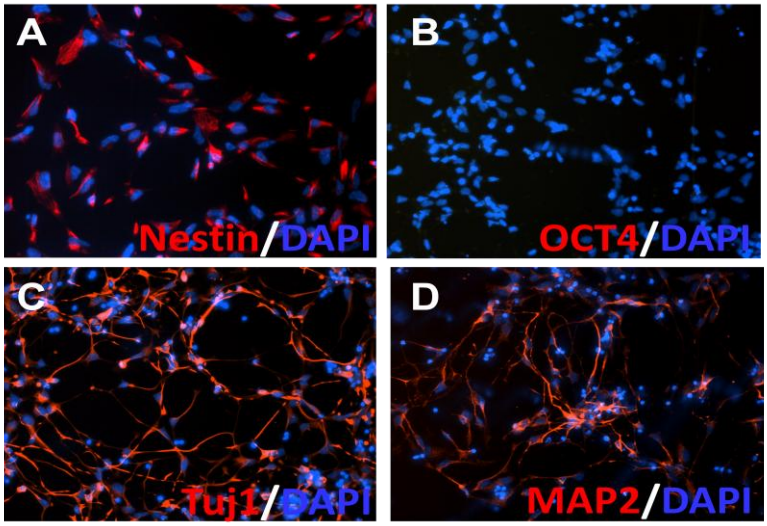

Fig 4: Using ArunA's adherent monolayer culture technology, generated iPSC-hNP cells resemble hNPs in morphology, stable proliferation (>10 passages), nestin (**A**) expression and loss of OCT4 expression (**B**). Following 2 weeks differentiation, iPSC-hNPs show neurite outgrowth and Tuj1 (**C**) and MAP2 (**D**) expression.

High throughput cell membrane proteomic screening and flow cytometry studies previously conducted by the Stice lab identified ciliary neurotrophic factor receptor alpha (CNTFRα) as a novel cell surface marker to distinguish hNP cells (59% CNTFRα+) from their WA09 hESC parent line (1% CNTFRα+) (**Table 1**) (*4*). CNTFRα and cytokine CNTF are members of the interleukin-6, leukemia inhibitory factor, and ciliary neurotrophic factor (IL-6/LIF/CNTF) receptor-ligand superfamily involved in Jak-STAT signaling pathways and implicated in regulating neural proliferation and lineage differentiation as well as CNS injury response (*5-6*). We therefore investigated whether CNTFRα is an effective selection marker for ArunA's hNP1 cells and iPSC-hNP1 cells. We found that ArunA's hNP1 cells and iPSC-hNP1 cells show distinct populations of CNTFRα positive cells, 73% and 38%, respectively (**Table 2**). We hypothesize that either CNTFRα positive or negative neural progenitors will show enhanced capacity for neural differentiation. In future studies we plan to determine the differentiation potential of CNTFRα positive and negative populations and use CNTFRα to separate by FACS those cells intended for genetic modification. By combining both CNTFRα selection and genetic modification, we hope to further expedite the rapid formation of electrically active and functional human neural networks.

Table 2. Flow Cytometry Results for CNTFRα Cell Surface Marker Expression

Cell Line	% CNTFRα +
iPSC Parent Line (generated w/viPS™ Vector Kit)	4
iPSC-hNP Cell Line 1	38
WA09 hESC Parent Line	1
ArunA's hESC-hNP Cells (STEMEZ™hNP1™ Cells)	73

To explore genetic modification of hNP1 cells to enhance neural differentiation, we have also assembled a collection of plasmids useful for further studies in Tasks 1 and 2. Our collection of plasmids is shown in **Table 3**. We have been currently working on transforming competent E. coli and creating plasmid stocks for use in these studies. One of the plasmids, pZsGreen1-N1, we have used as a control GFP vector to optimize plasmid transfection methods to explore genetically modifying hNP cells without the need for integrating lentiviral vectors. **Table 4** demonstrates the transfection efficiency of hNP cells utilizing various common commercial transfection reagents, as determined by flow cytometry. Utilizing Clontech's Xfect™ Transfection Reagent resulted in the highest transfection efficiency of ArunA's hNP1 cells, yet some cell death was apparent. Agilent's GeneJammer® showed the next highest transfection efficiency in ArunA's hNP1 cells without apparent loss of cell viability (**Fig 5**).

Table 3. Plasmids accumulated for genetic modification studies

Plasmid	Gene of Interest	Function
pCS2+ mNeurogenin1 Stu (mNgn1)	Neurogenin 1 (Ngn1)	bHLH transcription factor; neuronal differentiation
pCS2+ mNeurogenin2 (mNgn2)	Neurogenin 2 (Ngn2)	bHLH transcription factor; neuronal differentiation
pCS2+ mNeurogenin3 (mNgn3)	Neurogenin 3 (Ngn3)	bHLH transcription factor; neuronal differentiation
pCS2+ mNeuroD1	Neurogenic Differentiation 1 (BETA2/NeuroD1)	bHLH transcription factor; neuronal differentiation
pcDNA3 P38 eGFP pP38 mCherry	Synaptophysin (P38)	Synaptic vesicle glycoprotein with four transmembrane domains weighing 38kDa
pCMV PSD-95 eGFP pCMV PSD-95 mCherry pECFP PSD-95 CFP	Discs, large homolog 4 (PSD-95)	Post synaptic density protein; post-synaptic marker
pKv4.2-eGFP	Potassium voltage-gated channel subfamily D member 2 (Kv4.2)	Voltage-gated A-type potassium ion channel
pNICE Neuroligin 1 - YFP (NLGN1)	Neuroligin 1 (NLGN1)	Brain-specific, post synaptic membrane protein
pNICE Neuroligin 2 - YFP (NLGN2)	Neuroligin 2 (NLGN2)	Brain-specific, post synaptic membrane protein
pNICE Neuroligin 3 - YFP (NLGN3)	Neuroligin 3 (NLGN3)	Brain-specific, post synaptic membrane protein
pNICE Neuroligin 4 - YFP (NLGN4)	Neuroligin 4 (NLGN4)	Brain-specific, post synaptic membrane protein
pNICENeurexin1 beta – CFP	Neurexin-1-beta (NRXN1)	role in synaptogenesis; interacts with neuroligins
pBS Wnt-7aHA	Wingless-type MMTV integration site glycoprotein 7a (Wnt-7a)	Secreted glycoprotein important in developmental processes; modulates synaptic transmission
pCS2+Frizzled 9	Frizzled-9	Receptor for Wnt proteins in developmental processes
pCSM Bassoon-95-3938 -GFP conjugated	Bassoon (Bsn)	Pre-synaptic cytomatrix protein
pRSET-B tdTomato	tandem dimer Tomato red protein derived from DsRed (tdTomato)	Red fluorescent protein; reporter
pZsGreen1-N1	Human codon-optimized variant of Zoanthus sp. green fluorescent protein	Green fluorescent protein; reporter

Table 4. Comparison of Transfection Efficiencies of pZsGreen1-N1 Using Different Reagents

Transfection Reagent	% GFP +	Comments
Fugene HD	22	
Lipofectamine LTX	11	Much Cell Death
TurboFect	5.7	Extreme Cell Death
Xfect	69	Some Cell Death
TransIT	18	
GeneJammer	31	

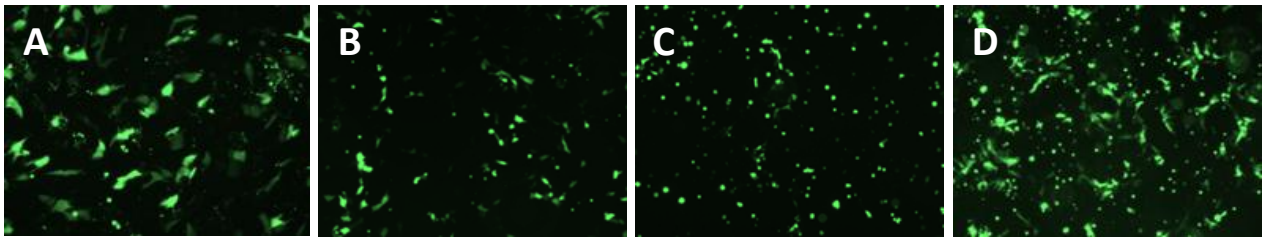

Fig 5: Epifluorescent images (10X objective) of hNP cells transfected with pZsGreen1-N1 control GFP plasmid using either GeneJammer(A), Fugene HD (B), Lipofectamine LTX (C) or Xfect (D) transfection reagents.

Concurrently, we assessed lentiviral transduction in ArunA's hNP1 cells to determine if both the EF1α and CMV promoters would show robust expression in order to be appropriate promoters to use in constructing bHLH lentiviral vectors. We observed that ArunA's hNP1 cells were very amenable to transduction with VSV-G pseudotyped lentiviral vectors with either the CMV or EF1α promoter (**Fig 6**). The hNP cells can also be transduced with lentiviral vectors with MOI's as low as 1 and 5 to yield GFP+ cell populations for greater than 10 passages. Transduction and expression from these lentiviral vectors encoding specific bHLH transcription factors should therefore not pose a problem in upcoming studies.

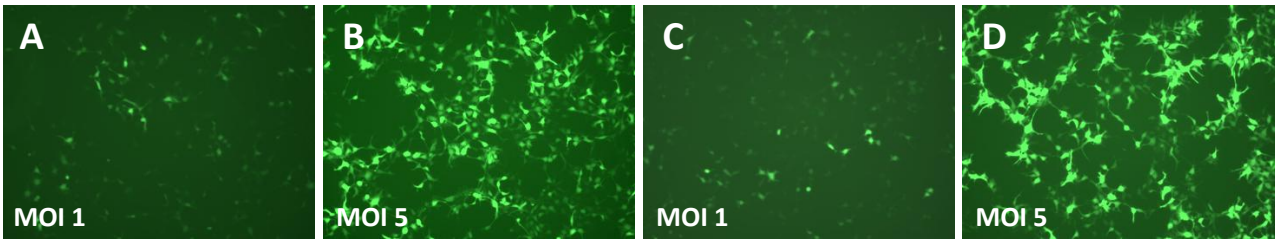

Fig 6: Epifluorescent images (10X objective) of hNP cells transduced with VSV-G pseudotyped lentiviral vectors with either the CMV (A-B) or EF1α promoters (C-D) at MOI 1 and 5.

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Aruna Biomedical

Job #7015	DNR DoD Biosensor	**Cost:**	**899,084.00**
Agency	ONR	**Fee:**	**62,916.00**
Phase	II	**Amount:**	**962,000.00**
Contract #	N00014-10-C-0066		
Period of Performance:	12/11/09		
	12/11/10		

		Jan- Mar '10	2010 Year To Date	Dec '09	Project To Date	Dec '09	Estimate to Complete
DIRECT LABOR		$32,009.76	$32,009.76	$7,054.07	$39,063.83	$7,054.00	$181,582.81
DIRECT LABOR FRINGE	22.97%	$7,352.64	$7,352.64	$1,620.32	$8,972.96	$1,620.30	$41,710.04
DIRECT LABOR OVH	46.14%	$18,161.81	$18,161.81	$4,002.36	$22,164.17	$3,254.72	$103,027.52
CONTRACT LABOR		$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
TOTAL LABOR & OVH		$57,524.21	$57,524.21	$12,676.75	$70,200.96	$11,929.02	$326,320.37
CONSULTANTS		$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
EQUIPMENT		$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
MATERIAL & PARTS		$28,996.54	$28,996.54	$1,649.94	$30,646.48	$1,649.94	$265,636.52
OTHER DIRECT COSTS		$538.44	$538.44	$0.00	$538.44	$0.00	$11,161.56
SUBCONTRACTS		$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
TRAVEL		$0.00	$0.00	$0.00	$0.00	$0.00	$5,000.00
TOTAL OTHER DIR. COST		$29,534.98	$29,534.98	$1,649.94	$31,184.92	$1,649.94	$281,798.08
TOTAL COSTS BEFORE G&A		$87,059.19	$87,059.19	$14,326.69	$101,385.88	$13,578.96	$608,118.45
GEN. & ADMIN. (G&A)	27.78%	$24,185.04	$24,185.04	$3,979.96	$28,165.00	$0.00	$161,414.67
TOTAL COST		$111,244.23	$111,244.23	$18,306.65	$129,550.88	$13,578.96	$769,533.12
FEE/PROFIT	7.00%	$7,785.34	$7,785.34	$1,281.18	$9,066.51	$0.00	$53,849.49
REVENUE		$119,029.57	$119,029.57	$19,587.82	$138,617.39	$13,578.96	$823,382.61

Estimate of Costs to Complete *$823,382.61*